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The capsid protein p38 of *turnip crinkle virus* is associated with the suppression of *cucumber mosaic virus* in *Arabidopsis thaliana* co-infected with *cucumber mosaic virus* and *turnip crinkle virus*

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ABSTRACT

Infection of plants by multiple viruses is common in nature. *Cucumber mosaic virus* (CMV) and *Turnip crinkle virus* (TCV) belong to different families, but *Arabidopsis thaliana* and *Nicotiana benthamiana* are commonly shared hosts for both viruses. In this study, we found that TCV provides effective resistance to infection by CMV in *Arabidopsis* plants co-infected by both viruses, and this antagonistic effect is much weaker when the two viruses are inoculated into different leaves of the same plant. However, similar antagonism is not observed in *N. benthamiana* plants. We further demonstrate that disrupting the RNA silencing-mediated defense of the *Arabidopsis* host does not affect this antagonism, but capsid protein (CP or p38)-defective mutant TCV loses the ability to repress CMV, suggesting that TCV CP plays an important role in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with both viruses.

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Introduction

Natural infection of plants by two or more plant viruses is a common phenomenon and can result in various effects, such as antagonism, synergism or coexistence. Synergism is a type of interaction in which co-infection by two or more different plant viruses can induce more severe symptoms than single infection, and this phenomenon is most often observed in interactions between unrelated viruses (Zhang et al., 2001; Choi et al., 2002). In synergistic interactions, in addition to the disease symptoms, the titers, movement, or both may be enhanced for one or both viruses. For instance, *Potato virus Y* (PVY) has been demonstrated to significantly enhance the replication and symptoms of several viruses, including *Potato virus X* (PVX), as well as *Cucumber mosaic virus* (CMV) in the well-studied PVY/PVX or PVY/CMV interactions (Rochow and Ross, 1955; Goodman and Ross, 1974a, 1974b; Vance, 1991; Vance et al., 1995; Pruss et al., 1997; Ryang et al., 2004; Mascia et al., 2010). Mixed infection of CMV and *Turnip mosaic virus* (TuMV) can induce more severe symptoms in *N. benthamiana* than single infection, but local interference between the two viruses can be detected even in the synergism (Takeshita et al., 2012). In contrast with synergism, mixed infection of two or more viruses can cause different degrees of antagonism (Bennett, 1951; Aguilar et al., 2000). In this phenomenon, the activity of a virus in a plant prevents or significantly reduces the expression of a subsequent challenge virus, which has been shown to be a strategy that can be used to control several viral diseases, including protection of crops from potyviral diseases, as well as CMV (Fulton, 1986; Sherwood, 1987; Aguilar et al., 2000). This phenomenon often occurs in unrelated viruses from different families or two closely related viruses belonging to one genus, including both RNA and DNA viruses, but the mechanism remains elusive (Kurihara and Watanabe, 2003; Owor et al., 2004; Kamei et al., 1969; Otsuki and Takebe, 1976).

Several mechanisms have been proposed to explain interactions between viruses. It is well established that in plants, multiple regulatory and defensive reactions are mediated by RNA silencing, which is a sequence-specific host defense mechanism against viral invaders (Brodersen and Voinnet, 2006; Voinnet, 2009). To combat this major line of plant defense, viruses have generally evolved various viral suppressors of RNA silencing (VSRs) that have distinct modes of action in the RNA silencing machinery of host plants (Voinnet et al., 1999). Many VSRs have been demonstrated to disturb the host gene-silencing machinery and induce various

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malformed phenotypes and developmental defects when expressed in transgenic plants (Mallory et al., 2002; Chapman et al., 2004; Dunoyer et al., 2004; Zhang et al., 2006; Shiboleth et al., 2007; Lewsey et al., 2007; Siddiqui et al., 2011). In many cases, the interactions between viruses are associated with the function of VSRs. It has been suggested that VSRs have important roles in tissue invasion patterns in mixed virus infections. The class 1 RNase III protein encoded by *Sweet potato chlorotic stunt virus* (SPCSV), which is a VSR, has the ability to break down resistance to *Sweet potato feathery mottle virus* (SPFMV) by eliminating the antiviral defense in sweet potato plants (Cuellar et al., 2009). The strong VSR helper component proteinase (HC-Pro), encoded by PVY, plays a key role in enhancing the accumulation of PVX in mixed infections (Brigneti et al., 1998; Vance, 1991). This synergistic effect also occurs in the interactions between PVX and other unrelated viruses, including *Tobacco vein mottling virus* (TVMV), *Tobacco etch virus* (TEV), and *Plum pox virus* (PPV) (Vance et al.,1995; Sáenz et al., 2001; Yang and Ravelonandro, 2002).

CMV (genus *Cucumovirus*, family *Bromoviridae*) and *Turnip crinkle virus* (TCV) (genus *Carmovirus*, family *Tombusviridae*) belong to different families, and both of them are among the relatively few viruses that are highly virulent on *Arabidopsis* (Van Regenmortel et al., 2000; Cohen et al., 2000). CMV 2b is one of the best characterized VSRs and has complex activities to suppress RNA silencing, control host basal resistances, and operate synergistic interactions with other viruses in both a virus- and a host-specific manner (Palukaitis and García-Arenal, 2003; Ding et al., 1994, 1995; Wang et al., 2004). It is well established that CMV can cause synergistic infections with *Tobacco mosaic virus* (TMV) in tomato and tobacco plants, and 2b protein of a mild strain of CMV (e.g., Kin) alone is sufficient to cause synergistic interaction with TMV, resulting in filiformic leaves which completely lack leaf blades in tobacco (Garces-Orejuela and Pound, 1957; Matthews, 1991; Bazzini et al., 2007; Cillo et al., 2009; Ye et al., 2009; Siddiqui et al., 2011). TCV can cause antagonistic interactions with infection of *Tobacco necrosis virus* (TNV) or CMV (Xi et al., 2010; Yang et al., 2010). In the present study, the interaction between CMV and TCV was investigated. We found that the infection of CMV is strongly suppressed by TCV, and the capsid protein (CP or p38) of TCV plays an important role in the resistance to CMV in *Arabidopsis* plants co-infected with CMV and TCV.

Results

The infection of CMV is strongly suppressed by TCV in Arabidopsis plants

CMV and TCV belong to different families. *A. thaliana* is a commonly shared host for both viruses, in which they can proceed systemic movement and induce markedly different symptoms (Van Regenmortel et al., 2000; Cohen et al., 2000). As shown in Fig. 1(a), in Col-0 plants, CMV infection exhibited moderate stunting with reduced petioles, and the newly emerging leaves were strongly distorted and clustered, whereas TCV induced strong symptoms such as obvious chlorosis and then progressed to severe vascular wilt and plant death. However, when CMV and TCV (T+C) were simultaneously inoculated onto the same leaf of *Arabidopsis* plants, the plants only developed strong chlorosis in the inoculated leaves and upper leaves which were the typical TCV-induced symptoms at 15 days post inoculation (dpi). A similar phenomenon was also observed in the sequential inoculations with CMV 3 days after TCV (T-C), indicating that CMV-induced symptoms are strongly suppressed by TCV. But in the sequential inoculations with TCV 3 days after CMV (C-T), both chlorosis and distorted leaves were observed, showing that symptoms induced by CMV are relatively unaffected in CMV pre-infected plants.

To test the interaction between CMV and TCV at different growth stages in Col-0 plants, viral RNAs extracted from inoculated leaves (IL) and systemic leaves (SL) at 7 and 12 dpi were analyzed by Northern blot. In three repeated experiments, the accumulation of CMV in the T+C or T-C inoculation was much lower as compared with that of single CMV infection at 12 dpi, even was below detection limits of Northern blot analysis at 7 dpi. However, in the C-T inoculation, CMV accumulation was similar to that of the CMV single infection in IL but was slightly lower in SL at 7 or 12 dpi, indicating that the systemic movement and replication of CMV is slightly suppressed by TCV (Fig. 1b–e). Subsequently, we analyzed the accumulation of TCV in CMV and TCV co-infected Col-0 plants. As shown in Fig. 1(b, c), at the early stage of mixed infection, the accumulation of TCV fluctuated somewhat. It may be that the level of antiviral defense against TCV varies during the course of infection. But at 12 dpi, the RNA levels of TCV in various inoculations were enhanced to a similar level in both IL and SL (Fig. 1d, e). These results are consistent with the symptoms induced by the two viruses, showing that the replication of TCV is not negatively affected by CMV, whereas TCV causes strong suppression to the replication and systemic movement of CMV in the T+C or T-C inoculation but mild suppression in the C-T inoculation. It is possible that TCV provides effective resistance against the infection of CMV in *Arabidopsis* plants co-infected with CMV and TCV, but this antagonistic effect can be overcomed by delaying the introduction of TCV for three days.

The relative locations on the plants of CMV and TCV inoculations affect the degree of the antagonistic effect

It has been shown that the relative locations of Fny-CMVΔ2b and Fny-CMV inoculations in tobacco plants affect the degree of cross-protection (Ziebell et al., 2007). Therefore, experiments were conducted in which Col-0 plants were co-inoculated with CMV and TCV on different leaves. Symptom development was monitored for 15 days after the first inoculation. When CMV and TCV were co-inoculated on different leaves of the same plant, the plants developed obviously different disease symptoms compared with co-inoculation on the same leaf. As shown in Fig. 2(a), in the T+C or T-C inoculation, the plants not only developed strong chlorosis, which was similar to the symptoms of TCV alone, but also exhibited mild distorted and clustered phenotypes, comparable to the symptoms of CMV alone. To further confirm this result, the RNA levels of TCV and CMV in SL were analyzed by Northern blot, and the accumulation levels of TCV in doubly infected Col-0 plants were found to be similar to those of plants singly infected with TCV at 7 dpi (Fig. 2b, c) and then increased to higher accumulation levels at 12 dpi (Fig. 2d, e). In contrast, at the early stage of infection, they were detectable but lower CMV accumulation in the T+C inoculation, which was not detected when CMV and TCV were inoculated on the same leaf (Fig. 2b, c). Five days later, although the accumulation levels of CMV in various mixed inoculations were all significantly increased, the titers were still much lower than those of CMV single infection (Fig. 2d, e). These results suggest that the systemic movement and replication of CMV in *Arabidopsis* plants is suppressed by TCV when the two viruses are inoculated on different leaves but the degree of antagonistic effect is less than when they are inoculated on the same leaf.

Host plants affect the interaction between CMV and TCV

The model plants *A. thaliana* and *N. benthamiana* are well-adapted plant hosts of CMV and TCV (Qu and Morris, 1999; Hou et al., 2011).

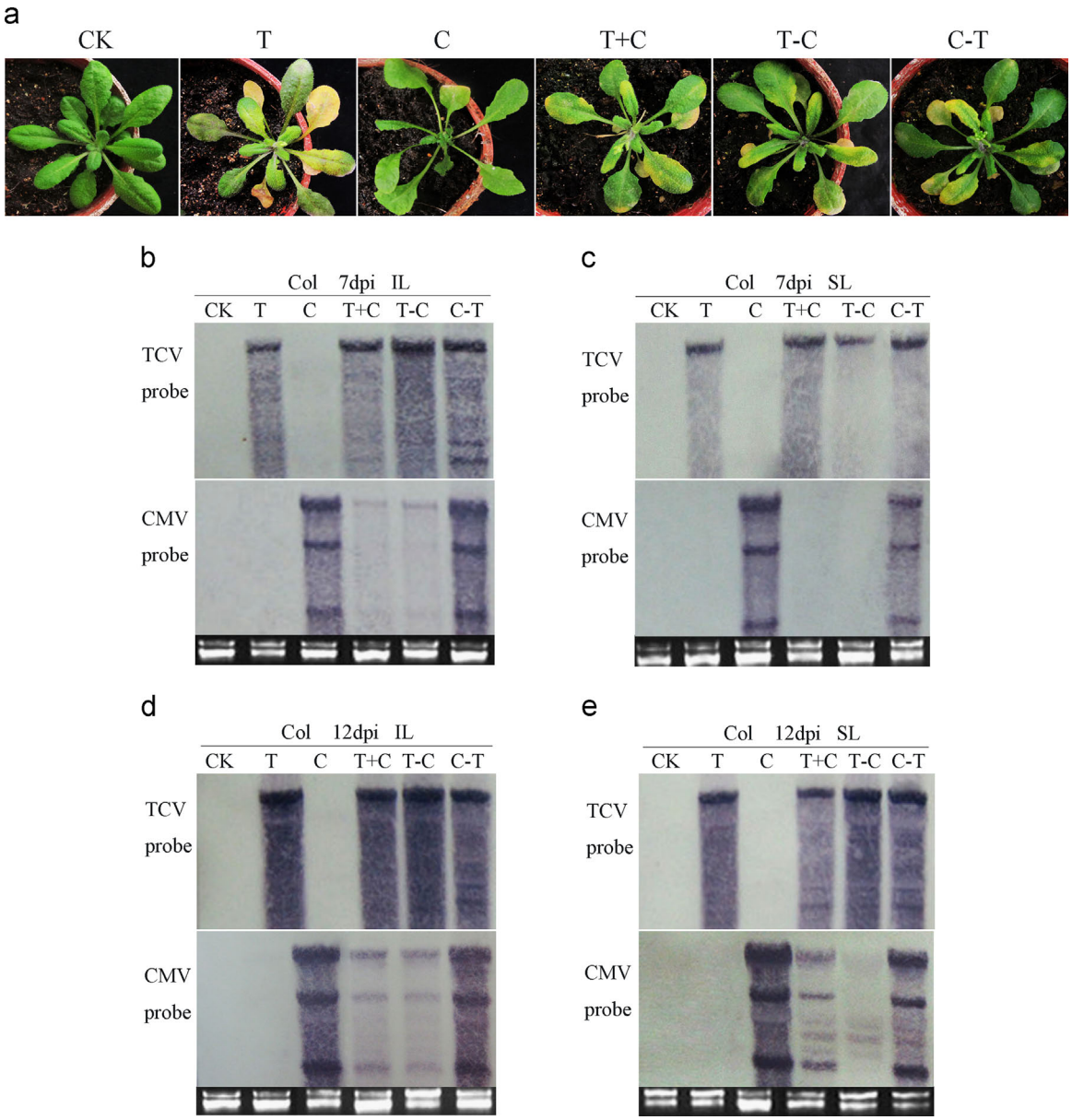

Fig. 1. Symptoms induced by CMV and TCV in either singly or mixed infected Col-0 plants and accumulation levels of the two viruses when they were inoculated on the same leaf. (a) Symptoms on Col-0 plants infected with CMV and TCV. Plants were photographed for 15 days after the first inoculation. CK, buffer-inoculated; T, plants inoculated with TCV; C, plants inoculated with CMV; T+C, plants inoculated with CMV and TCV simultaneously; T-C, plants inoculated with CMV 3 days after TCV; C-T, plants inoculated with TCV 3 days after CMV. (b–e) Northern blot showing the accumulation of viral RNAs in Col-0 plants. (b) and (c), the RNA levels of CMV and TCV in IL (b) and SL (c) at 7 dpi. (d) and (e), the RNA levels of CMV and TCV in IL (d) and SL (e) at 12 dpi. IL and SL indicate inoculated leaves and systemic leaves, respectively.

The same virus combination may have an entirely different outcome in another host (Sherwood, 1987). Thus, the host plant (*N. benthamiana*)-viruses (CMV and TCV) system was also established to investigate the interaction between CMV and TCV in *N. benthamiana* plants. As shown in Fig. 3(a), in *N. benthamiana* plants, CMV infections induced leaf curling and natural drooping tendencies whereas TCV infections continued to develop obvious mosaic phenotypes in newly emerging leaves for about two weeks. Malformations characteristic of both infections were evident in doubly infected plants. However, the mosaic phenotype induced by TCV in the C-T inoculation was milder than that of TCV single inoculation or other mixed inoculations. Nevertheless, typical CMV-induced symptoms were similar in various mixed infections. The results in *N. benthamiana* and Col-0 plants show significant differences.

To detect the presence of CMV and TCV in infected *N. benthamiana* plants, Northern blot was used to analyze total RNA extracted from IL and SL (Fig. 3b–e). At the early stage of mixed infection, in the T-C inoculation, the accumulation of TCV was strongly enhanced compared with that of TCV single infection, but there was a small reduction in the C-T inoculation (Fig. 3b, c). Although the RNA levels of TCV in various mixed inoculations fluctuated substantially at 7 dpi, they were enhanced to a similar level in both IL and SL at 12 dpi, except for a slight decrease in the sequential inoculations (C-T) in SL (Fig. 3d, e). It is possible that CMV can induce mild suppression of the systemic movement of TCV when CMV is initially inoculated to *N. benthamiana* plants. CMV titers were unexpectedly strongly enhanced in CMV and TCV co-infected *N. benthamiana* plants, but there was still a delayed accumulation of CMV in the T-C inoculation at 7 dpi (Fig. 3b, c). 5 days later, CMV titers in various inoculations were significantly enhanced to a similar level, which was never observed in Col-0 plants (Fig. 3d, e). It is possible that local interference occurs at the early stage of mixed infection in *N. benthamiana* plants. Overall, these results indicate that the interaction between CMV and TCV may be significantly affected by host factors.

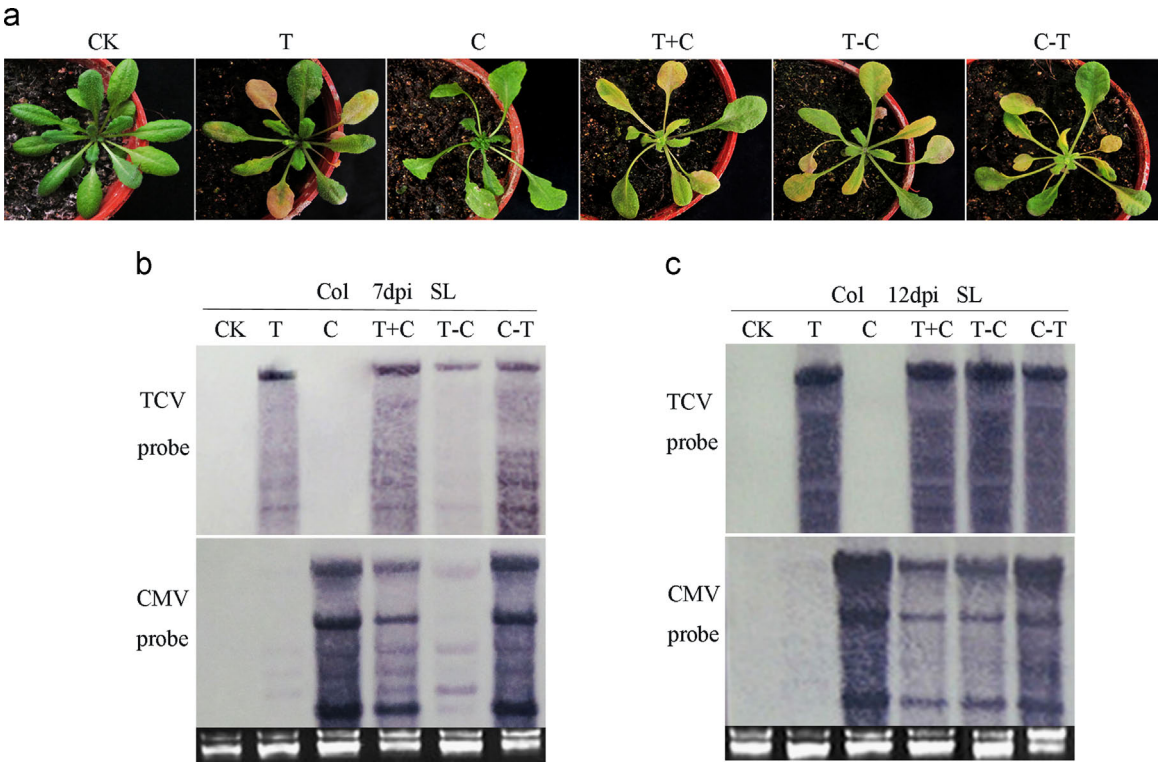

Fig. 2. Symptoms of Col-0 plants infected singly with CMV or TCV, or doubly with CMV and TCV on different or adjacent leaves and accumulation levels of the two viruses. (a) Symptoms on Col-0 plants infected with CMV and TCV. Plants were photographed for 15 days after the first inoculation. (b–c) Northern blot showing the accumulation of viral RNAs in SL of Col-0 plants infected with CMV and TCV at 7 dpi (b) and 12 dpi (c).

Host RNA silencing does not affect the antagonistic effect of TCV toward CMV

RNA silencing is thought to be associated with the viral antagonism phenomenon known as cross-protection and also with synergism (Brodersen and Voinnet, 2006; Voinnet, 2009). To further investigate the role of RNA silencing in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with those two viruses. Experiments conducted with mutant *Arabidopsis* plants (*dcl2*/*dcl3*/*dcl4*) compromised in silencing machinery may provide a definitive answer (Deleris et al., 2006). As shown in Fig. 4(a), in CMV and TCV singly or doubly infected *dcl2*/*dcl3*/*dcl4* mutant plants, TCV-induced symptoms showed no significant differences; however, typical CMV-induced symptoms were observed only in the C-T inoculation and CMV single inoculation, indicating that plants initially infected with TCV are resistant to subsequent infection by CMV. It seems that the disease symptoms induced by CMV and TCV in *dcl2*/*dcl3*/*dcl4* mutant plants are similar to those in Col-0 plants. Next, the levels of accumulated viral RNAs in single and mixed infections were examined. Northern blot analysis showed no significant differences between the changes in TCV and CMV titers in *dcl2*/*dcl3*/*dcl4* and Col-0 plants (Fig. 4b–e compared with Fig. 1b–e). These results suggest that disrupting the RNA silencing-mediated defense of the *Arabidopsis* host does not affect the antagonistic effect of TCV toward CMV. It is possible that a strong suppressor encoded by wild-type TCV effectively masks the antiviral role of silencing pathway genes of the host (Qu et al., 2008).

TCV CP plays a key role in the antagonistic effect of TCV toward CMV in Arabidopsis plants

In this study, we demonstrated that TCV provides effective resistance against infection by CMV in *Arabidopsis* plants. It has been suggested that viral VSRs have important roles in tissue invasion patterns in mixed virus infections (Cuellar et al., 2009; Brigneti et al., 1998; Vance, 1991). TCV CP is a silencing suppressor and plays a significant role in the infection phenotype. Thus, we speculated that TCV CP is involved in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with CMV and TCV. To test this hypothesis, a CP-defective mutant TCV, TCVΔCP, was constructed (Fig. 5b). In *Arabidopsis*, DCLs are involved in antiviral RNA silencing, and *dcl2*/*dcl3*/*dcl4* triple mutants with inactivated DCL2, DCL3 and DCL4 are necessary and sufficient to restore systemic infection of TCVΔCP (Deleris et al., 2006; Cao et al., 2010). Next, CMV and TCVΔCP were inoculated onto *dcl2*/*dcl3*/*dcl4* mutant plants to investigate the role of TCV CP in the antagonistic effect of TCV toward CMV. As shown in Fig. 6(a), in *dcl2*/*dcl3*/*dcl4* mutant plants, TCVΔCP induced strong chlorosis, like wild-type TCV, for about four weeks, and then progressed to severe vascular wilt and plant death. Similar symptoms were also observed in various mixed infected plants. As expected, CMV-typical symptoms of distorted and clustered leaves in various mixed infected plants were as severe as in CMV singly infected plants. That is, when CMV and TCVΔCP were co-inoculated onto the same leaf of *dcl2*/*dcl3*/*dcl4* mutant plants, the infected plants induced CMV- and TCV-typical symptoms simultaneously. Northern blot was used to further confirm this result. At the early stage of infection, the accumulation of TCVΔCP in various mixed infections was slightly lower than that of TCVΔCP single infection in IL (Fig. 6b), but at 12 dpi, TCVΔCP titers in single and mixed inoculations were all enhanced to a similar level in both IL and SL, except for a slight decrease in the C-TCVΔCP inoculation (Fig. 6d, e). Nevertheless, at 7 dpi, CMV accumulation in the TCVΔCP+C inoculation was strongly enhanced, but its accumulation in the TCVΔCP-C inoculation was still much lower, indicating a delayed accumulation of CMV (Fig. 6b, c). But 5 days later, CMV titers in all plants were significantly enhanced to a similar level (Fig. 6d, e). Similar results

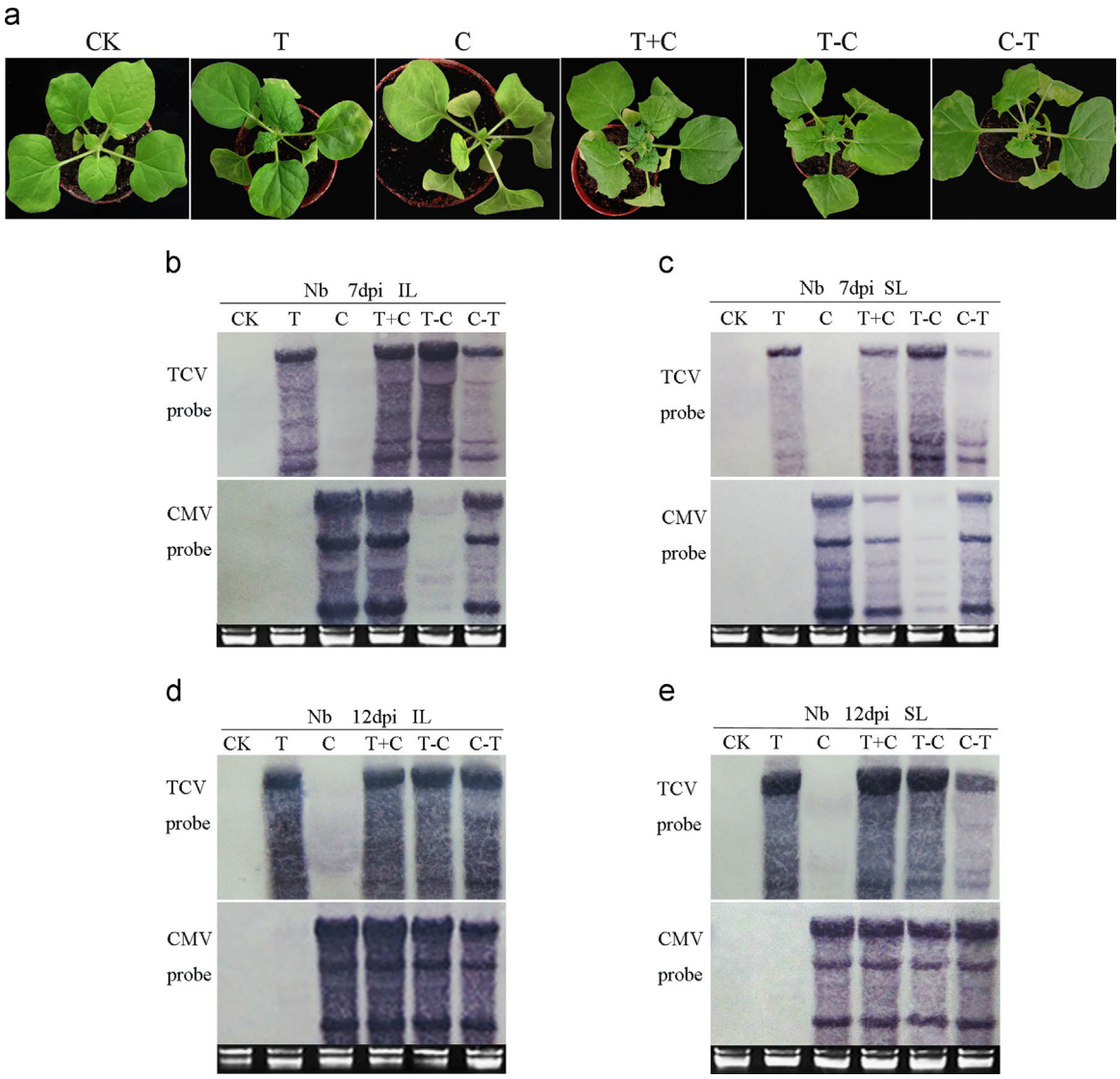

Fig. 3. Typical CMV- and TCV- induced symptoms in either singly or doubly infected *N. benthamiana* plants and accumulation levels of the two viruses when they were inoculated on the same leaf. (a) Typical symptoms induced by CMV and TCV on *N. benthamiana* plants. Plants were photographed for 15 days after the first inoculation. (b–e) Northern blot showing the accumulation of viral RNAs in *N. benthamiana* plants. (b) and (c), the RNA levels of CMV and TCV in IL (b) and SL (c) at 7 dpi. (d) and (e), the RNA levels of CMV and TCV in IL (d) and SL (e) at 12 dpi.

also occurred in *N. benthamiana* plants but were never observed in Col-0 plants. Overall, these results suggest that the CP-defective mutant TCV loses the ability to repress CMV, that is, TCV CP plays a key role in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with CMV and TCV.

TCV CP is a multifunctional protein needed for virus assembly and suppression of RNA silencing-based antiviral defense in infected plants. To further confirm the role of TCV CP in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants, a silencing suppressor-minus TCV mutant (TCV-TH) containing two-amino-acids substitutions was generated by reference to Cao et al. (2010) (Fig. 5c). Then, CMV and TCV-TH were co-inoculated onto *dcl2*/*dcl3*/*dcl4* mutant plants, and viral RNAs extracted from IL and SL at 12 dpi were analyzed by Northern blot. As shown in Fig. 7, at 12 dpi, similar accumulation levels of CMV RNAs in single and mixed inoculations in both IL and SL were observed. These results were consisitent with the results of Fig. 6, indicating that the silencing suppression function of TCV CP plays an important role in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants.

Discussion

In this study, we found that the expression of CMV is significantly suppressed in *Arabidopsis* plants co-infected with CMV and TCV, whereas the expression of TCV seems to be unaffected (Fig. 1). However, this antagonism is most effective when the two viruses are co-inoculated on the same leaf of *Arabidopsis* plants. It was found that the location of the challenge inoculation site relative to the site inoculated with the first virus had a clear effect on the degree of cross-protection (Ziebell et al., 2007). Thus, when CMV and TCV were inoculated on different leaves of the same plant, all the plants displayed typical symptoms induced by both TCV and CMV simultaneously but CMV-induced symptoms in mixed infections were milder than those of CMV single infection (Fig. 2a). That is, the degree of antagonism is weaker, suggesting that it may be related to the competition occurring on inoculated leaves. Some of our findings appear to be consistent with a model for Fny-CMVΔ2b-mediated cross-protection (Ziebell et al., 2007; Ziebell and Carr, 2009). It is possible that when two viruses are inoculated on the same leaf, the presence of the first virus may

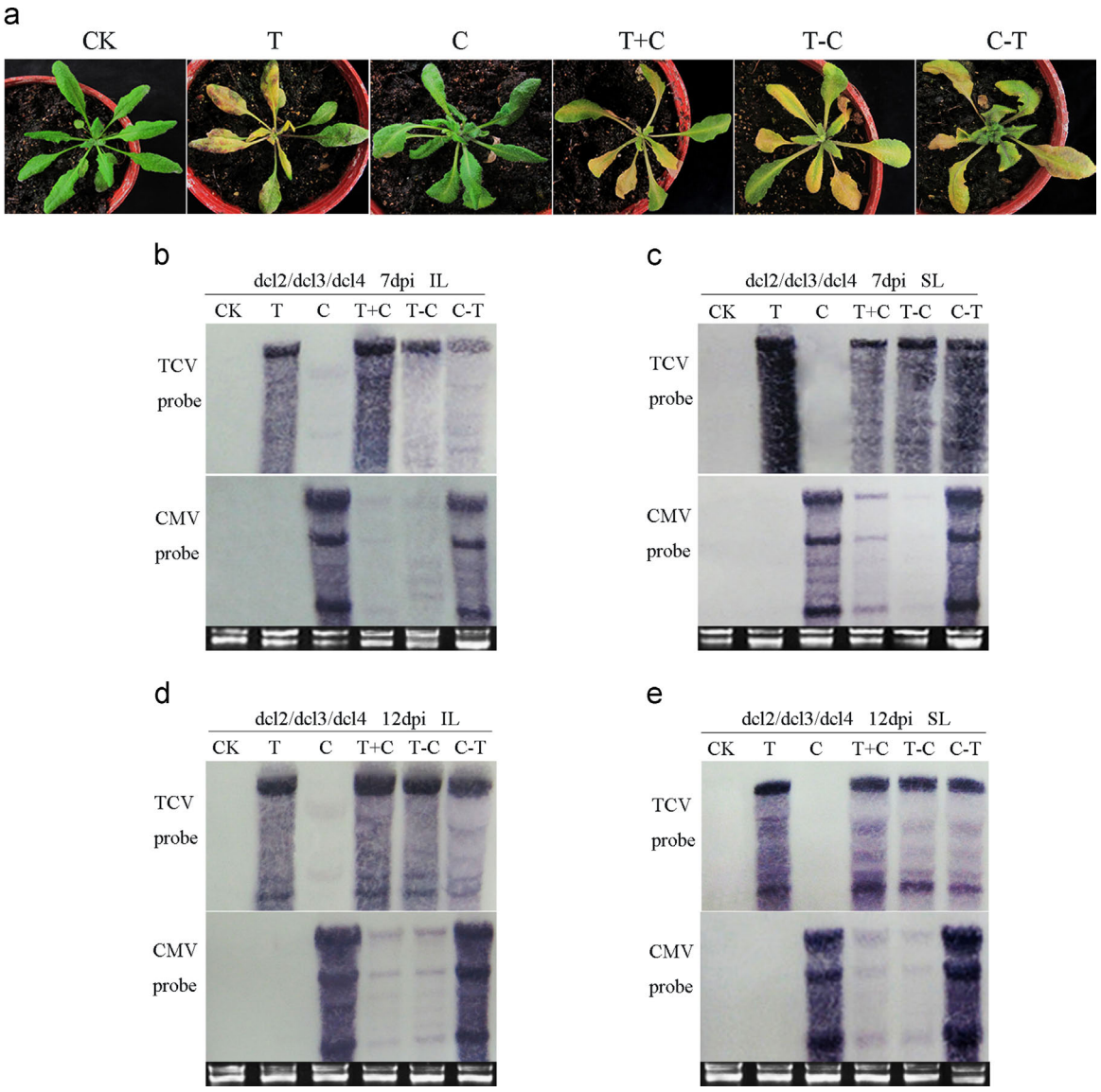

Fig. 4. Symptoms of *dcl2*/*dcl3*/*dcl4* mutant plants infected singly with CMV or TCV, or doubly with CMV and TCV on the same leaf and accumulation levels of the two viruses. (a) Typical symptoms induced by CMV and TCV on *dcl2*/*dcl3*/*dcl4* mutant plants. Plants were photographed at three weeks after the first inoculation. (b–e) Northern blot showing the accumulation of viral RNAs in *dcl2*/*dcl3*/*dcl4* plants. (b) and (c), the RNA levels of CMV and TCV in IL (b) and SL (c) at 7 dpi. (d) and (e), the RNA levels of CMV and TCV in IL (d) and SL (e) at 12 dpi.

significantly reduce the susceptibility of the plant to infection by the second virus by excluding the second virus from cells that have been occupied or by competing for host factors needed by the second virus (Ziebell and Carr, 2009). We demonstrated that Col-0 plants initially infected with TCV are resistant to subsequent infection by CMV. Interestingly, however, the replication of TCV is not significantly suppressed in Col-0 plants initially infected with CMV, which appears to be partly inconsistent with the model of exclusion, although competition remains possible.

Although the infection of CMV is strongly suppressed by TCV in Arabidopsis plants co-infected with CMV and TCV, this phenomenon cannot be reproduced in *N. benthamiana* plants. When *N. benthamiana* plants were co-infected with CMV and TCV, both viruses moved to and accumulated in young, developing tissues, resulting in additive effects of the symptoms, different from those caused by each virus alone (Fig. 3). These results suggest that the host plant may play an important role in the interaction between CMV and TCV. Host-dependent patterns have also been reported for plants co-infected with PVX and a potyvirus (González-Jara et al., 2004), *Tomato chlorosis virus* (ToCV) and *Tomato infectious chlorosis virus* (TICV) (Wintermantel et al., 2008), and *Pepper huasteco virus* (PHV) and *Pepper golden mosaic virus* (PepGMV) (Méndez-Lozano et al., 2003). The pattern of virus–virus and viruses-host interactions suggests the existence of differences between viruses in adaptation to different hosts, and these differences may eventually translate into competitiveness of each virus in doubly infected host plants.

RNA silencing is one of the potent mechanisms of antagonism (cross-protection), using small RNA molecules (21–30 nt in length) as sequence-specific mediators to regulate the expression of a diverse array of genes including invasive viruses, viroids or mobile RNA-transposable elements (Ratcliff et al., 1997, 1999; Voinnet et al., 1999; Voinnet, 2001; Waterhouse et al., 2001). When plants are co-infected with two viruses, one virus overwhelms the other virus through RNA-mediated resistance if both viruses share a nucleotide sequence (Ratcliff et al., 1999). Most examples of antagonism are found between two closely related viruses (Gal-On and Shiboleth, 2006; Hull, 2002; Ratcliff et al., 1999). However, many viruses belonging to different families have also been demonstrated to induce antagonistic effects, though the mechanism of antagonism between distinct plant viruses

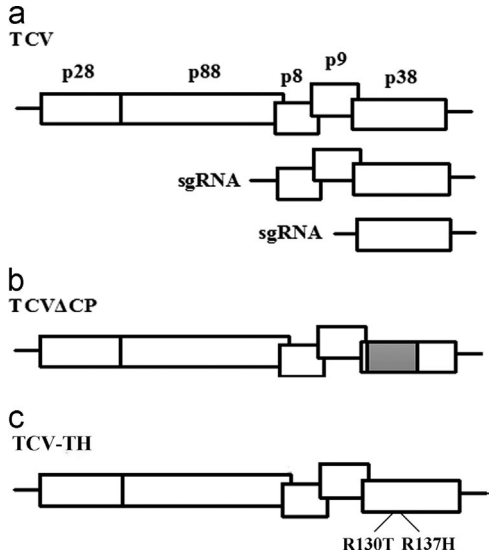

Fig. 5. Schematic presentation of the genome structure of wild-type TCV and its mutant TCVΔCP. (a) Wild-type TCV contains five open reading frames (ORFs) including two replicases (p28, p88), two MPs (p8, p9), and a CP (p38 or CP), and two sgRNAs of 1.7 and 1.45 kb. (b) Diagram of the TCVΔCP construct used in this study. Wild-type regions were shown as open boxes. The mutated regions are shown as gray boxes. (c) Diagram of the TCV-TH construct used in this study. The exact amino acid changes in the TCV-TH mutant were marked with thick line.

remains elusive (Bazzini et al., 2007; Xi et al., 2010; Yang et al., 2010). Antagonism is complicated by the fact that interactions between plants and viruses are multifaceted, and different viruses have a number of patterns of interaction within an infected plant. To investigate the role of RNA silencing in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with CMV and TCV, experiments were conducted with mutant plants compromised in the silencing machinery (*dcl2*/*dcl3*/*dcl4*). Using this system, we demonstrated that disrupting the RNA silencing-mediated defense of the *Arabidopsis* host does not affect this antagonism (Fig. 4). The 2b of CMV and CP of TCV are well characterized VSRs that act on different stages of RNA silencing (Palukaitis and García-Arenal, 2003; Qu et al., 2003). Although RNA silencing is a potent antiviral defense in plants, well-adapted plant viruses are known to encode VSRs that can neutralize the effectiveness of RNA silencing (Diaz-Pendon et al., 2007; Qu et al., 2008; Zhang et al., 2012). It is possible that a strong silencing suppressor encoded by wild-type TCV effectively masks the antiviral role of silencing pathway genes of the host (Qu et al., 2008). Consistent with our findings, Kurihara and Watanabe (2003) showed that in *Arabidopsis* RDR6 is not required for the antagonism between two Tobamovirus spp., indicating that RNA silencing does not play a major role in this interaction. Furthermore, Ziebell and Carr (2009) also reported that general antagonism is not completely dependent on RNA silencing but may be considered as simple competition between these viruses.

In mixed infections, VSRs have been demonstrated to play important roles in tissue invasion patterns (Cuellar et al., 2009; Brigneti et al., 1998; Vance, 1991). CMV 2b functions as a symptom determinant and is known to inhibit systemic transport of the silencing signal into newly developing leaves, but it cannot suppress the effect of RNA silencing machinery that is established in plant tissues before virus invasion (Brigneti et al., 1998; Takeshita et al., 2012). It has been demonstrated that CMV can cause synergistic infections with a number of other viruses (Wang et al., 2004; Takeshita et al., 2012). A special case of viral synergism is known to occur in tomato and tobacco plants co-infected with CMV and TMV in which the 2b silencing suppressor of CMV alone is sufficient for synergistic interaction with TMV and induction of severe leaf malformation in 2b-transgenic tobacco plants (Garces-Orejuela and Pound, 1957; Matthews, 1991). This result represents the other example in which the severe synergistic effect between two viruses is due to a single viral protein. However, in the interaction between CMV and TCV in *Arabidopsis* plants, the expression of CMV is significantly suppressed. TCV CP is a multifunctional protein and has a dramatic effect on the infection phenotype, leading to rapid tissue necrosis and plant death. Thus, the role of TCV CP in the antagonistic effect of TCV toward CMV was investigated by using the mutant TCVΔCP and TCV-TH to infect the *dcl2*/*dcl3*/*dcl4* triple mutants with inactivated DCL2, DCL3 and DCL4 which are necessary and sufficient to restore systemic infection of TCVΔCP and TCV-TH (Deleris et al., 2006). Surprisingly, we found that the silencing suppressor p38 of TCV plays a key role in the antagonistic effect in *Arabidopsis* plants. But the silencing suppressor-minus TCV mutant (TCV-TH) containing two-amino-acids substitutions, to some extent, may affect virion assembly (Cao et al., 2010). Therefore, virion assembly function of TCV CP may also be involved in the suppression of TCV toward CMV in *Arabidopsis* plants. Together, our data suggest that the capsid protein p38 of TCV plays an important role in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with both viruses. Nevertheless, how TCV CP interferes with the replication of CMV still needs further study. Jeong et al. (2008) showed that the interaction between TCV CP and the NAC transcription factor TIP protein is likely important in the basal defense response to TCV. Donze et al. (2014) further confirmed the role of TCV CP in inducing basal resistance. It is possible that the antagonism associated with TCV CP may be explained by the ability of TCV to induce basal resistance in *Arabidopsis* plants. Although the TCV CP plays a key role in the antagonistic effect of TCV toward CMV in *Arabidopsis* plants co-infected with CMV and TCV, our present data still do not allow us to exclude the possibility that simple competition between the two viruses for host factors is also involved in this process.

Materials and methods

Plant materials

The *Arabidopsis* mutant was in the Col-0 background. The seed stock number was as follows: triple mutant plants *dcl2*/*dcl3*/*dcl4* (CS 16391) had been described previously (Hammond, 2005; Deleris et al., 2006), and were ordered from Arabidopsis Biological Resource Center. All Arabidopsis plants and *N. benthamiana* seeds were reared in a growth room set at 20–22 °C, with 12 h of daylight. When there were six to eight true leaves in *Arabidopsis* and *N. benthamiana* plants, the plants were inoculated with various viruses.

Construction of recombinant TCV and plant infection

The infectious cDNA clone of TCV (previously known as T1d1) was kindly provided by Dr Anne E Simon (Department of Cell Biology and Molecular Genetics, University of Maryland, USA). A CP-defective mutant TCV, TCVΔCP, was constructed by deleting the fragment (nt 2752 to 3387) from the CP gene of TCV infectious clone. This was achieved by overlap extension PCR (Higuchi et al., 1988) with a pair of primers (5′- GAAATGGAAAATGCACCTACGGCCAAGGAGC -3′; and 5′- GGCCGTAGGTGCATTTTCCATTTCCAGTGTTG - 3′). A silencing suppressor-minus TCV mutant (TCV-TH) containing two-amino-acids substitutions was generated by reference to Cao et al. (2010). This was also achieved by overlap extension PCR (Higuchi et al., 1988) with a pair of primers (5′- ACGTTCACGTCAC TCAGATTTCACTACTCTC - 3′; 5′- GTGAAATCTGAGTGACGTGAACGTGTATTT - 3′). To test whether the recombinant TCV was infectious, the viral clone was linearized with *Sma*I, and the linearized DNA served as a template for in vitro

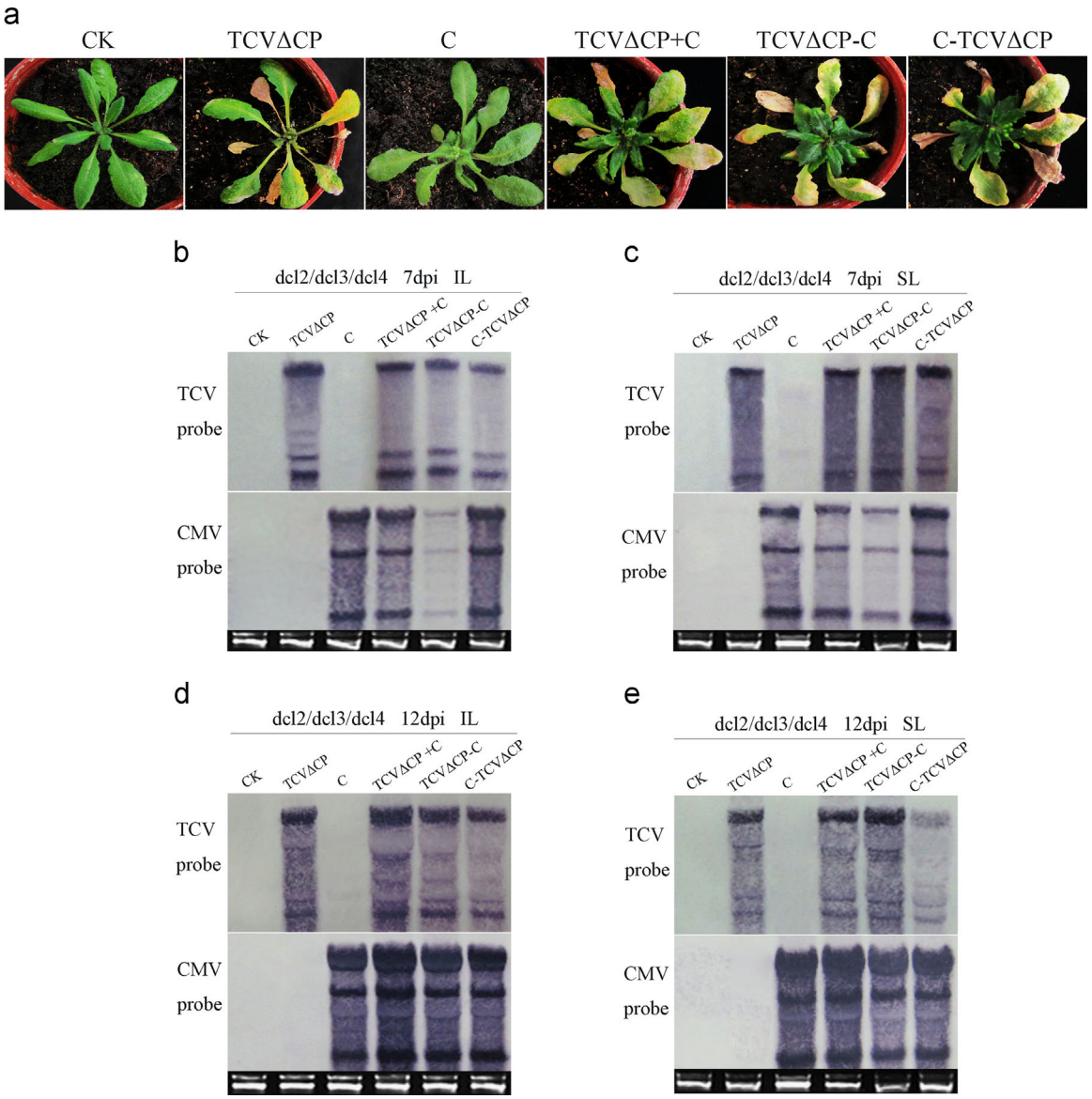

Fig. 6. Typical symptoms induced by CMV and TCVΔCP in either singly or doubly infected *dcl2*/*dcl3*/*dcl4* plants and accumulation levels of the two viruses when they were inoculated on the same leaf. (a) Symptoms on *dcl2*/*dcl3*/*dcl4* plants infected with CMV and TCVΔCP. Photographs were taken for four weeks after the first inoculation. CK, buffer-inoculated; TCVΔCP, plants inoculated with TCVΔCP; C, plants inoculated with CMV; TCVΔCP+C, plants inoculated with CMV and TCVΔCP simultaneously; TCVΔCP-C, plants inoculated with CMV 3 days after TCVΔCP; C-TCVΔCP, plants inoculated with TCVΔCP 3 days after CMV. (b–e) Northern blot showing the accumulation of viral RNAs in *dcl2*/*dcl3*/*dcl4* plants. (b) and (c), the RNA levels of CMV and TCVΔCP in IL (b) and SL (c) at 7 dpi. (d) and (e), the RNA levels of CMV and TCVΔCP in IL (d) and SL (e) at 12 dpi.

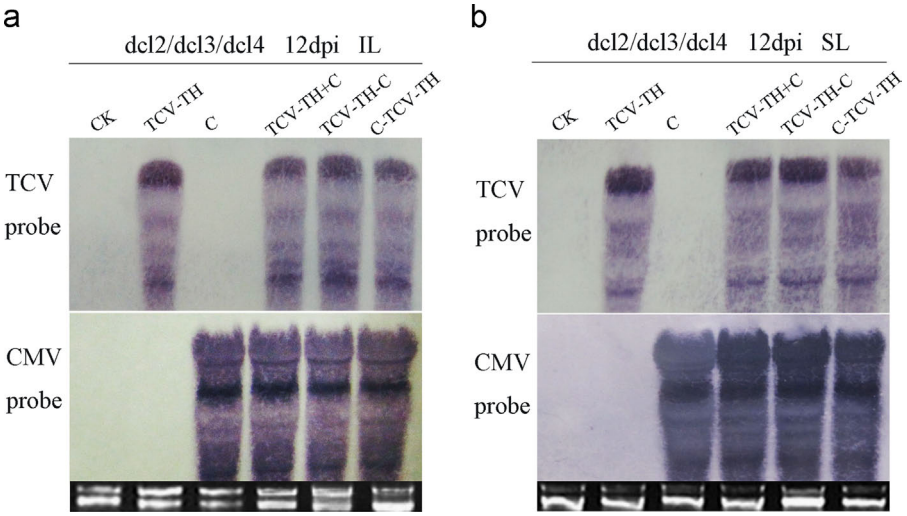

Fig. 7. Northern blot showing the accumulation of viral RNAs in *dcl2*/*dcl3*/*dcl4* plants. (a) and (b), the RNA levels of CMV and TCV in IL (a) and SL (b) at 12 dpi. CK, buffer-inoculated; TCV-TH, plants inoculated with TCV-TH; C, plants inoculated with CMV; TCV-TH+C, plants inoculated with CMV and TCV-TH simultaneously; TCV-TH-C, plants inoculated with CMV 3 days after TCV-TH; C- TCV-TH, plants inoculated with TCV-TH 3 days after CMV.

transcripts for inoculation. All the *Arabidopsis* and *N. benthamiana* plants were inoculated with the first virus at the six-to-eight leaf stage. After a period of 3 days, some of the plants were challenged by the second virus. To minimize experimental variations, for Col-0 and *dcl2*/*dcl3*/*dcl4* mutant plants, eight IL and SL from eight different plants infected with the same inoculum were collected at 7 dpi and 12 dpi; For *N. benthamiana* plants, four IL and SL from four different plants infected with the same inoculum were also collected at 7 dpi and 12 dpi.

RNA extraction and northern blot analysis

Total RNAs were extracted from collected samples with a Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) and the RNA samples were subjected to RNA blot hybridization with DIG-labelled probes (DIG-High Prime DNA Labeling and Detection Starter Kit I, Roche Molecular Biochemicals), according to the manufacturer's instructions. Both CMV- and TCV-specific RNAs were detected by hybridization with DIG-labelled probes corresponding to the 3′untranslated region. The probe for CMV was synthesized by PCR from CMV infectious cDNA clone with a pair of primers (5′- GGTGAACGGGTTGTCCATCC -3′; and 5′- TGGTCTCCTTTTAGAGACC -3′) to detect CMV viral RNAs. The probe for TCV was synthesized by PCR from TCV infectious cDNA clone with a pair of primers (5′–GGAAAGATCTGCCGGTCTCG-3′; and 5′–CAGGCC CCCCCCCCGCGCGA-3′) to detect TCV viral RNAs.

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